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(54) Title: APPARATUS AND METHOD FOR CHARACTERIZING A SAMPLE BY AN OPTICAL PULSE

(57) Abstract: An apparatus for characterising a sample, such as a flow cytometer or a microscope, comprises a laser, in particular a laser configured to emit solitons, for irradiating the sample with an optical pulse and an optical detector for detecting fluorescence radiation emitted by the sample as a consequence of said irradiation. The sample is excited by multiphoton excitation or a nonlinear optical device is inserted between the laser and the sample for frequency conversion, thereby obviating the use of a UV laser. The apparatus optionally comprises pulse shaping elements like a pulse stretcher, a pulse amplifier, and a pulse compressor. Light transmission between the laser and the sample may be via optical fibres, in particular optical telecommunication fibres, wherein the light pulse may be distributed to a plurality of measurement heads by means of one or more optical multiplexers.

APPARATUS AND METHOD FOR CHARACTERIZING A SAMPLE BY AN OPTICAL PULSE

Field of the Invention

This invention relates to apparatus and methods for characterizing a sample by an optical pulse.

Background of the Invention

Biological materials such as proteins and cells can be characterised by interrogating them with short optical pulses and analysing the resulting fluorescence with analytical instruments. Similar techniques are also applied to the characterisation of many other materials. Equipment utilizing such techniques can be found in confocal microscopes, flow cytometers, and many other analytical equipment used in the life science, medical and analytical industries. Applications include the characterization of proteins, gene recognition and labelling, and the characterization and sorting according to sex of live biological materials such as sperm for breeding purposes.

One significant limitation of such instruments is scattering from the sample its matrix, support, coverslip and/or the flow sheaf the sample is in. In flow cytometry a jet of fluid 100 microns or more across is made to leave an orifice at speeds in excess of 10m/s. This is illuminated by a laser and particles passing down the laser interrupt the laser beam. It will be obvious to one in the art that the amount of light scattered by such a fluid sheaf is significant and this typically disallows

the use of light scattering information for small particles. Fluorescence is the preferred option for analysis of small particles. Fluorescence is the emission of Stoke shifted light, which is due, at least in part, to optical excitation. A Stokes shift is defined as an emission of photons that has lower energy then the photons used to excite the sample. If the case of 2 photon excitation the Stokes shift defines that the energy of the emitted photons is less than that of 2 photons of the excitation light and so forth for higher orders of multiple photons. Fluorescent emission may be due to an irreversible process.

However in many fluorophores commonly used the Stokes shift is small and to allow efficient filtering of the fluorescent signal a significant dark signal passes the fluorescence filter due to scatter. The detectors used are typically PMT's (Photon Multiplier Tubes) and thus more sensitive to shorter wavelengths and thus the dark noise rather than the signal itself. It is a limitation of fluorescence that the input energy is of the same form as the output energy and thus filters are required to remove the excitation light which may be orders of magnitude greater than the emission and said filters attenuate the emitted light even where a large stokes shift exist. It is further limitation that the filters may require to be replaced if a differing flurophore or laser is required. Another type of detector, which may be used, is a solid state detector. Solid state detectors include avalanche photodiodes.

One method of detection of a fluorescent species is by means of what is known in the art as a pump probe

experiment where one laser of a first wavelength excites the sample then a second laser of a longer wavelength is directed at the sample and the sample acts as an amplifier increasing the pulse. One limitation of the pump probe technique is the requirement to phase-lock the two pulsed sources. A third limitation of the technique is the requirement to rapidly gate a detector if the first detector is to ignore the excitation pulse.

The preferred laser is a UV laser. UV lasers are however very expensive, consume significant quantities of electrical power and a significant infrastructure is required in order to remove heat generated from what is often the inefficient generation of the UV light. UV light is known to produce ozone, which is damaging to equipment. It is known in the art that UV is highly attenuated by common glasses and plastics used in biochemical laboratories and specialised expensive optics must be used. It is known in the art that UV light is strongly attenuated by fluids. Thus UV excitation of water based samples introduces significant losses. Typically a continuous wave laser is used for which a suitable emission line is around 360nm. A typical laser is an Argon Ion gas laser, which is highly inefficient especially at UV wavelengths where it emits a plurality of laser lines between 350 and 365nm. The lasers are typical rated at between 1 and 5W, where the rating is given for visible light. They are typically in excess of 4 feet long and of the order of 1 foot wide by 1 foot high. They take 3 phase power (35A rated on 3 phases) and require water cooling of the order of 10 litres a minute. The water should be a closed loop to stop fouling or scale build up. Water and high power electricity make them dangerous

especially when failure appears as well as being expensive in running costs of the power the consumed parts are highly expensive. A laser gas tube is of the order of £20,000 and lasts a few thousand hours.

Typically, powers between 50 and 500mW are used and this requires a large laser operating from a three phase power supply, requiring water cooling.

It is known in the art that UV light is damaging to tissue and may cause genetic damage and thus should not be used as an investigative tool of animals or people or biological materials (such as sperm) used for breeding purposes.

In most measuring instruments involving a laser, the laser power must be set. This is typically carried out by reducing the pump laser emission or by using filters to reduce the final harmonic generated emission. Both methods have serious disadvantages: reducing the laser below saturation leads to increase variation in laser emission, and increased power on the harmonic crystal leads to reduced crystal life.

It is an aim of the present invention to obviate or reduce at least one problem of the prior art, whether one of the above-mentioned problems or not.

Summary of the Invention

According to one aspect of the present invention, there is provided apparatus for characterizing an optical emission of a sample interrogated by a first optical pulse, which

apparatus comprises a laser and an instrument, and in which the apparatus is configured such that the first optical pulse illuminates the sample, and the instrument is configured to characterize the optical emission.

This enables the provision of an apparatus for distributing short pulse laser emissions to a plurality of measurement heads.

The apparatus may comprise a detector for detection of the optical emission, and the apparatus may be configured such that the illumination and detection are confocal.

The instrument may comprise a flow cytometer. The instrument may comprise a microscope. The instrument may be confocal.

The sample may comprise materials which include but are not excluded to a biological material, a tissue sample, a protein, a cell, a section of a protein, a section of DNA, a section of RNA, a chromosome, a gene, a sperm cell, a sperm celled dyed on order to allow determination of its sex or an antibody, an animal or person. The sample may contain a chemical material. The same may comprise an inorganic material or a component.

The apparatus may contain a detector, which is blind to the wavelength of the laser and thus does not suffer the problems of scatter leakage.

The laser may be a semiconductor laser, a Nd: Yag laser, a Nd glass laser, or a fibre laser. The fibre laser may comprise a rate-earth dopant selected from the group

Ytterbium, Erbium, Neodymium, Praseodymium, Thulium, Samarium, Holmium, and Dysprosium.

The laser may be a mode-locked laser. The laser may comprise a continuous wave laser and an optical switch. The laser may emit the first optical pulse having a first wavelength, a second optical pulse having a second wavelength, or emit pulses of two or more wavelengths.

The apparatus may comprise at least one non-linear device for frequency conversion of the second optical pulse into the first optical pulse.

The non-linear device may comprise a non-linear crystal. The non-linear crystal may be Potassium Titanyl Phosphate (KTP) crystal. The non-linear device may comprise a parametric oscillator.

The non-linear device may be an optical switch, a poled-fibre, a KTP or other crystalline wave guide, a non-linear optical loop mirror, a Kerr gate, an optical fibre, a non-linear amplifying loop mirror, or a non-linear optical modulator.

The non-linear device may be a holey fibre. The holey fibre may comprise glass. The glass may be silica, a silicate glass, or a compound glass.

The holey fibre may comprise a core and a cladding, in which the cladding comprises a plurality of holes arranged around the core, and in which the core has a diameter less than 10um. The core may have a diameter less than 5um. The core may have a diameter less than 2um.

The holey fibre may comprise a dopant. The dopant may be selected from the group comprising Ytterbium, Erbium, Neodymium, Praseodymium, Thulium, Samarium, Holmium, Dysprosium, Tin, Germanium, Phosphorous, Aluminium, Boron, Antimony, Bismuth, Lead, a transition metal, and a semiconductor.

The non-linear device may be, or may comprise, a semiconductor optical amplifier. The non-linear device may comprise a lithium niobate wave guide. The wave guide may be either a channel wave guide, or a planar wave guide.

The non-linear device may comprise a periodically poled lithium niobate channel wave guide. The wave guide may be either a channel wave guide, or a planar wave guide.

The optical non-linearity used within the non-linear optical device may be based on second-order ($x(2)$), or third-order ($x(3)$) non-linear effects. The specific manifestations/use of the non linearity might be in terms of Self-Phase Modulation (SPM), Cross-Phase Modulation (CPM), Four-Wave Mixing (FWM), parametric frequency conversion, second harmonic generation, third harmonic generation, cascaded second order effects, or some combination thereof. Other optical non-linearities that might be used include Raman and Brillouin effects, cross gain modulation and two photon absorption.

The apparatus may comprise a pulse stretcher. The pulse stretcher may comprise an optical circulator and a fibre

Bragg grating. The pulse stretcher may comprise a dispersive element.

The apparatus may comprise a pulse compressor. The pulse compressor preferably comprises a bulk grating. The pulse compressor may comprise an optical circulator and a fibre Bragg grating. The pulse stretcher may comprise a dispersive element.

The apparatus may further comprise an optical amplifier. The optical amplifier may be an optical fibre amplifier comprising a rare-earth dopant selected from the group Ytterbium, Erbium, Neodymium, Praseodymium, Thulium, Samarium, Holmium and Dysprosium. The optical fibre amplifier may be one based on Erbium Ytterbium codoping.

The apparatus may comprise a transmission fibre. The transmission fibre may be an optical telecommunication fibre. The transmission fibre may be designed to minimize dispersion. The transmission fibre may be configured to minimize non-linear effects such as self phase modulation.

The apparatus may comprise at least one first multiplexer.

A multiplexer is a device which can switch light between a plurality of outputs or share light between a plurality of outputs according to a pre-defined ratio. It is known in the art that such devices may be polarisation maintaining or polarisation sensitive.

The apparatus may comprise at least one second multiplexer.

The laser may be configured to emit short pulses. The laser may be configured to emit solitons. The laser is preferably a mode-locked fibre laser. The mode-locked fibre laser may comprise a rate-earth doped optical fibre comprises a rare earth dopant. The rare-earth dopant is preferably erbium. The laser preferably emits in the 1550nanometre telecommunication window.

The laser may be configured to be remote from the sample.

The instrument may comprise an optical detector configured to be remote from the sample.

Suitably, the apparatus comprises a sample handler for receiving the first optical pulse, in which the sample handler is connected to the laser by an optical cable having a length of at least 50 metres.

Suitably, the optical cable has a length of at least 5 metres. Suitably, the optical cable has a length of at least 20 metres.

Suitably, the first multiplexer multiplexes the received optical pulse to a plurality of sample handlers.

Suitably, the instrument comprises a non-linear crystal, which instrument is distant from the laser.

Suitably, the laser has a repetition rate set to ensure that each sample is illuminated by at least 50 laser flashes during its dwell time in the beam. Suitably, the laser has a repetition rate set to ensure that each sample

is illuminated by at least 100 laser flashes during its dwell time in the beam.

Suitably, the laser is operated at or near saturation, the apparatus comprises a fibre for conveying light to a non-linear crystal and the magnitude of the light emitted to a sample is controlled by obstructing some of the light from the fibre from being incident on the non-linear crystal. Suitably, the obstruction is by a variable opening iris.

Suitably, there is provided means for exciting the sample by multiphoton excitation.

Suitably, the multiphoton excitation is second order multiphoton excitation.

Suitably, the multiphoton excitation is third order multiphoton excitation.

Suitably, the multiphoton excitation is configured to occur within an effective area that is smaller than the area that the laser is focussed onto within the sample.

Suitably, the sample is defined by an effective wavelength characterised by single photon excitation and in which the sample is not penetrative to illumination at the effective wavelength, and in which the method comprises illuminating the sample at a wavelength that is penetrative to the sample using multiphoton excitation.

Suitably, the effective wavelength is less than 350nm, wherein the laser delivers UV light without formation of ozone.

Suitably, the instrument comprises a detector, and in which the detector has a higher gain at wavelengths emitted by the sample under test than the wavelength of the multiphoton excitation.

Suitably, the wavelength of the multiphoton excitation is in the region of 1500-1600nm.

Suitably, the wavelength of the multiphoton excitation is in the region of 970nm to 1140nm.

Suitably, the wavelength of the multiphoton excitation is in the region 750-900nm.

Suitably, the apparatus comprises a probe pulse to generate emission from a sample.

Suitably, the apparatus further comprises a short pulse generator for generating a short pulse is used to generate multiphoton excitation of the sample.

Suitably, the apparatus includes a pulse stretcher for stretching the short pulse.

Suitably, the apparatus includes an amplifier for amplifying the stretched short pulse, and a compressor for compressing the amplified stretched short pulse.

Suitably, the short pulse is a soliton.

Suitably, the sample is remote from the laser.

According to a second aspect of the present invention, there is provided a sperm sexing apparatus comprising apparatus for characterizing an optical emission of a sample according to the first aspect of the invention.

The invention also provides a method for analysing a sample comprising providing a laser, a sample, and an instrument for characterizing optical emission from a sample, and exciting the sample by multiphoton excitation.

The multiphoton excitation may be fourth order multiphoton excitation. The multiphoton excitation may be fifth order multiphoton excitation.

The multiphoton excitation may be configured to occur within an effective area that is smaller than the area that the laser is focussed onto within the sample.

The sample may be defined by an effective wavelength characterised by single photon excitation and in which the sample is not penetrative to illumination at the effective wavelength, and in which the method comprises illuminating the sample at a wavelength that is penetrative to the sample using multiphoton excitation.

The effective wavelength may be less than 350nanometres. This is particularly advantageous because UV excitation results in ozone generation, whereas multiphoton excitation would not result in the formation of ozone.

The instrument may comprise a detector having a higher gain at wavelengths emitted by the sample under test than the wavelength of the multiphoton excitation. The

detector may have very low responsivity at the wavelength of the multiphoton excitation.

The wavelength of the multiphoton excitation may be in the region of 1500-1600nm. The wavelength of the multiphoton excitation may be in the region of 970nm to 1140nm. The wavelength of the multiphoton excitation may be in the region of 750-900nm.

The method may be used to sex sperm from an animal. The animal may be livestock comprising one of cattle, pigs, sheep, and horses. The sperm may be used for artificial insemination of a female animal from the same species.

It is frequently beneficial to sort the sperm of livestock and use artificial insemination of livestock. For the purpose of this invention livestock may be defined as any animal that is kept, allowed to roam on lands or sold at least in part for commercial gain and may include but is not excluded to horses, bulls, pigs, sheep, goats, rabbits. In addition it may be beneficial to carry out similar means for animals such as pets and other animals that may not normally be considered livestock such as deer. Pet breeding is usually done for commercial reasons and thus dogs, cats and other species may be considered livestock in terms of this invention and that deer may generate finance by means of sale of hunting rights or of the animal and thus again may be considered livestock.

One method of use of this invention is by means of increasing the stag population of a deer herd above that which would be considered normal and thus allowing a

greater number of licences to be issued for the hunting of said stags leading to financial gain.

The method may include the step of providing a probe pulse to generate emission.

A preferred method is to provide a short pulse to generate multiphoton excitation of the sample. The method may include the step of stretching the short pulse. The short pulse may be amplified and then compressed. The short pulse may be a soliton.

The sample may be remote from the laser.

The method may comprise the steps of generating solitons, stretching the solitons, and amplifying the stretched solitons.

The method may be used to analyse a sample selected from the group comprising a biological material, a tissue sample, a protein, a cell, a section of protein, a section of DNA, a section of RNA, a chromosome, a gene, a sperm cell, an antibody, an animal and a person.

Brief Description of the Drawings

Embodiments of the invention will now be described solely by way of example and with reference to the accompanying drawings; in which:

Figure 1 shows apparatus according to the present invention for characterizing a sample by an optical pulse;

Figure 2 shows an apparatus according to the present invention in which the first optical pulse is generated by a non-linear device;

Figure 3 shows a holey fibre;

Figure 4 shows an apparatus according to the present invention and comprising a pulse stretcher;

Figure 5 shows an apparatus according to the present invention and comprising a first multiplexer;

Figure 6 shows an apparatus according to the present invention and comprising single photon excitation;

Figures 7 to 10 show apparatus according to the present invention and comprising multiphoton excitation;

Figure 11 illustrates an emission area; and

Figure 12 illustrates the use of a probe pulse.

Figure 13 illustrates an apparatus according to the present invention using a pump laser at saturation.

Figure 14 illustrates a sperm cytometer apparatus according to the present invention.

Description of Preferred Embodiments of the Invention

Figure 1 shows apparatus for characterizing an optical emission 5 of a sample 2 interrogated by a first optical pulse 4, which apparatus comprises a laser 1 and an

instrument 3, and in which the apparatus is configured such that the first optical pulse 4 illuminates the sample 2, and the instrument 3 is configured to characterize the optical emission 5.

The instrument 3 may comprise a flow cytometer. The instrument 3 may comprise a confocal microscope.

The sample 2 may comprise a biological material, a tissue sample, a protein, a cell, a section of a protein, a section of DNA, a section of RNA, a chromosome, a gene, or a sperm cell. The sample 2 may comprise an inorganic material or a component.

The laser 1 may be a semiconductor laser, a Nd:Yag laser, a Nd glass laser, or a fibre laser comprising a rare-earth dopant selected from the group Ytterbium, Erbium, Neodymium, Praseodymium, Thulium, Samarium, Holmium, and Dysprosium. By way of example a 355nm line can be produced by tripling a Yttrium Aluminium Garnate (YAG) laser. A 10W YAG laser emitting pulses at 80 MHz can produce 1W of UV light without rapidly damaging the crystal.

The laser 1 may be a mode-locked laser.

The laser 1 may emit the first optical pulse 4 having a first wavelength 11.

An alternative configuration is shown in Figure 2 in which the laser 1 emits a second optical pulse 20 having a second wavelength 22. The second optical pulse 20 is

frequency converted into the first optical pulse 4 by a non-linear device 21.

The non-linear device 21 may comprise a non-linear crystal such as KTP, periodically poled lithium niobate or a parametric oscillator.

The non-linear device 21 may be an optical switch, a poled-fibre, a KTP or other crystalline wave guide, a non-linear optical loop mirror, a Kerr gate, an optical fibre, a non-linear amplifying loop mirror, or a non-linear optical modulator.

The non-linear device 21 may be a holey fibre 30 an example of which is shown in figure 3. The holey fibre 30 may comprise glass. The glass may be silica, a silicate glass, or a compound glass.

The holey fibre 30 may comprise a core 31 and a cladding 32, in which the cladding 32 comprises a plurality of holes 33 arranged around the core31, and in which the core 31 has a diameter 34 less than 10um. The core 31 may have a diameter less than 5um (micrometres). The core 31 may have a diameter less than 2um.

The holey fibre 30 may comprise a dopant. The dopant may be selected from the group comprising Ytterbium, Erbium, Neodymium, Praseodymium, Thulium, Samarium, Holmium, Dysprosium, Tin, Germanium, Phosphorous, Aluminium, Boron, Antimony, Bismuth, Lead, a transition metal, and a semiconductor.

The non-linear device 21 may be, or may comprise, a semiconductor optical amplifier.

The non-linear device 21 may comprise a lithium niobate wave guide. The wave guide may be either a channel wave guide, or a planar wave guide.

The non-linear device 21 may comprise a periodically poled lithium niobate channel wave guide. The wave guide may be either a channel wave guide, or a planar wave guide.

The optical non-linearity used within the non-linear optical device may be based on second-order (χ^2), or third-order (χ^3) non-linear effects. The specific manifestation/use of the non-linearity might be in terms of Self-Phase Modulation (SPM), Cross-Phase Modulation (CPM), Four-Wave Mixing (FWM), parametric frequency conversion, second harmonic generation, third harmonic generation, sum frequency generation, difference frequency generation, super continuum generation, cascaded second order effects, or some combination thereof. Other optical non-linearities that might be used included Raman and Brillouin effects, cross gain modulation and two photon absorption.

Figure 4 shows a preferred embodiment of the invention, which comprises a pulse stretcher 42 and a pulse compressor 47. The apparatus may further comprise an optical amplifier 45 and/or a transmission fibre 46.

The pulse stretcher 42 stretches a pulse 41 emitted from the short-pulse laser 40 and outputs a lengthened pulse 48. The pulse stretcher 42 may comprise an optical

circulator 43 and a fibre Bragg grating 44. The fibre Bragg grating 44 is preferably chirped. The pulse stretcher 42 may comprise a dispersive element, which may be an optical fibre.

Stretching the pulse is particularly advantageous because it reduces the peak intensity and permits the output of the laser 40 to be transmitted over considerable distances (up to several tens of kilometres) without inducing non-linear effects in the transmission fibre 46.

The pulse compressor 47 outputs the second optical pulse 20. The pulse compressor 47 preferably comprises at least one bulk grating. Alternatively, the pulse compressor may comprise an optical circulator and a fibre Bragg grating. The pulse compressor 47 is preferably configured to counteract the dispersion in the pulse stretcher 42, the amplifier 45 and the transmission fibre 46.

The optical amplifier 45 may be an optical fibre amplifier comprising a rare-earth dopant selected from the group Ytterbium, Erbium, Neodymium, Praseodymium, Thulium, Samarium, Holmium, and Dysprosium.

The transmission fibre 46 may be an optical telecommunication fibre. The transmission fibre 46 may be designed to minimize dispersion.

Alternatively, parabolic amplifiers such as those disclosed in WO-A-01/86344 (Auckland Uniservices) may be used. Using the chirped pulse amplifiers (CPA) described above, the grating in front of the CPA spreads the pulse, which the CPA then amplifies. The pulse then has to be

recompressed with a grating pair. Using a parabolic amplifier, the pulse enters the amplifier as a short pulse and is chirped by the amplifier to provide an essentially linear chirp, which is then compressed by a grating pair. Methods of amplifying short pulses are described in US-A-6,104,249, which is hereby incorporated herein by reference.

Some of the advantages of the preferred embodiment of Figure 4 are illustrated in the arrangement of Figure 5, which comprises a first multiplexer 51 and a second multiplexer 52. This arrangement permits the pulse 41 to be shared between a plurality of samples 2.

The laser 40 may be configured to emit solitons. The laser 40 is preferably a mode-locked fibre laser and is preferably diode pumped. The technical papers "Passive, All-Fibre Source of 30 fs Pulses", Richardson et al, Electronics Letters 9 April 1992, Vol 28, No. 8, pp 778-779 and "Practical low-Noise Stretched-Pulse Yb^{3+}-Doped Fiber Laser", Lefort et al, Optics Letters 1 March 2002, Vol 27, No. 5, pp 291-293 provide examples of soliton generation from a passively mode-locked fibre laser operating in the 1550nm telecommunication window and a stretched-pulse fibre laser operating at around 1060nm. The laser 40 may have a pulse repetition rate between 1kHz and 100MHz (preferably 70-90MHZ and more preferably about 80MHz) and the apparatus is preferably configured such that there are multiple photons occurring at the peaks of the first optical pulses 5.

Mono-colour and multi-colour solitons, as well as continuous broadband spectra can be used (see for instance US-A-2002/0168161).

The laser used to analyse the sample has a repetition rate set to ensure that each sample is illuminated by at least 50 and preferably 100 laser flashes during its dwell time in the beam.

Thus, when the laser is operated at or near saturation the magnitude of the light emitted to the sample can be controlled by regulating the power from the fibre that is incident on the non-linear crystal.

A particular advantage of the preferred embodiments shown in Figures 4 and 5 is that the laser 40 may be configured to be remote from the sample 2. Moreover, it is possible to collect the optical emission 5 from the sample 2 with optical fibres such as holey fibres having a core diameter in the range 35um to 200um, but preferably in the range 75um to 110um. Thus the lasers and most of the instrumentation can both be located away from the sample 2. This arrangement has important consequences for many industrial and agricultural processes such as sperm characterization for artificial insemination where it is preferable to complete the characterization as quickly as possible after production.

As an alternative to the use of a harmonic crystal, multiphoton excitation can be utilised. For this a high peak power and a narrow laser beam are required.

Figure 6 shows single-photon excitation of a sample. A single photon 60 promotes an electron 61 from a first energy band 62 to a second energy band 63. At some time later, the electron emits a characteristic photon 69 as it decays to a third energy band 64. The third energy band 64 may be the same as the first energy band 61. There may also be an intermediary transition from the second energy band 63 to a fourth energy band 65.

Figure 7 illustrates two photon excitation of the sample by a first and a second photon 71, 72. The first and second photons 71, 72 preferably have the same wavelength, but may have different wavelengths. The sum of the energies of the first and second photons 71, 72 are equal to the energy of the single photon 60.

Figure 8 illustrates three photon excitation of the sample by a first, second and third photon 81, 82, 83. The sum of the energies of the first, second and third photons are equal to the energy of the single photon 60.

Figure 9 illustrates four photon excitation of the sample by a first, second, third and fourth photon 91, 92, 93, 94. The sum of the energies of the first, second, third and fourth photons 91, 92, 93, 94 are equal to the energy of the single photon 60.

Figure 10 illustrates five photon excitation of the sample by a first, second, third, fourth and fifth photon 101, 102, 103, 104, 105. The sum of the energies of the first, second, third, fourth and fifth photons 101, 102, 103, 104, 105 are equal to the energy of the single photon 60.

Two, three, four and five photon excitation are all examples of multiphoton excitation and in particular can be referred to as second, third, fourth and fifth order multiphoton excitation respectively. In each case, it is important that the electron 60 is excited to the second energy level 63 before it has time to decay - i.e. multiple photons need to arrive "essentially together" which is why short pulses such as solitons are particularly advantageous. The pulse width should be less than 10ps for two photon excitation. For three photon excitation less than about 500fs is required, typically 20fs - 500fs. The shorter the pulse width, the less energy per pulse that has to be provided - again indicating that solitons having temporal widths 30fs to 50fs are preferred sources.

The apparatus should be configured so that the multiple photons not only arrive together, but overlap to provide the required multiphoton excitation. Figure 11 shows the area 101 in which the laser is focussed onto the sample. The effective area 111 is the area from which the characteristic photon 69 is emitted. The effective area 111 may be smaller than the area 101.

The single photon 60 of Figure 6 has a wavelength, which can be defined to be the effective wavelength 68 of the sample. It is often the case that samples are not penetrative to optical illumination at the effective wavelength 68. Multiphoton excitation can be very advantageous in these circumstances if the sample is penetrative to these photons that have a longer wavelength.

The use of 1550nm short pulse sources (such as soliton sources) has particular advantages for microscopy since it permits analysis at greater depths in the sample.

The use of high order multiphoton sources has particular advantage in microscopy and confocal scanning as it allows a smaller effective emission focus.

The effective wavelength 68 may be less than 350nm. This is particularly advantageous because single photon - i.e. UV excitation results in ozone generation, whereas multiphoton excitation would not result in the formation of ozone.

The instrument may comprise a detector having a higher gain at wavelengths emitted by the sample under test than the wavelength of the multiphoton excitation. The detector may have very low responsivity at the wavelength of the multiphoton excitation.

The wavelength of the multiphoton excitation may be in the region of 1500-1600nm. The wavelength of the multiphoton excitation may be in the region of 970nm to 1140nm.

The wavelength of the multiphoton excitation may be in the region of 750-900nm.

At 8 MHz with a 100fs pulse width, the mean power required for three photon excitation is 200mW (milliWatts).

For sperm sexing applications two photon excitation can occur in the region 500-800nm where the light was generated by a laser emitting in the region of 1000nm -

1600nm. Similarly three photon excitation can occur in the region of 900nm - 1100nm.

Figure 12 illustrates the use of a probe pulse 120 to generate emission. The sample is excited using multiphoton excitation, and then illuminated by the probe pulse 120 at the emission wavelength of the characteristic photon 69 thus yielding two characteristic photons 69. This method is particularly advantageous if it is desired to control the direction of the characteristic emission (such as when characterising and/or sorting flat cells such as sperm cells) or to remove energy from samples that would not normally emit. The wavelength of the probe pulse can also be varied to study molecule photo-dynamics. The probe pulse can be locked to the multiphoton illuminating pulse. And the multiphoton source can be separated into different paths with the apparatus configured such that the multiple photons arrive at the sample in a time sufficient to ensure multiphoton excitation.

Referring to Figure 13, an IR laser 200 is coupled into a fibre by a lens arrangement 202.The fibre splits into numerous paths by means of a beam splitter 204. One each fibre output 206 the emitted light passes through a biconvex iris 208 to a biconvex lens 210. The lens refocuses the light on the harmonic crystal 212. Varying the biconvex iris size allows fine control of the laser power. A detector and a feedback loop may be used to stabilise the intensity. A detector and readout to the analysis programme may be used to compensate for changes in the laser intensity. The detector may be in two parts where one part is masked from the laser beam and use a

reference to allow accurate measurement by the second part. The laser 200 is run at saturation and the power deployed to the crystal 212 is controlled. This maximises power stability and crystal life.

Referring to Figure 14, there is shown a sperm cytometer 220 comprising a solid-state laser 222 as described above, connected via a fibre optic cable 224 to a cytometer head 226. The cytometer head 226 comprises a sample handler 228. In the embodiment shown the fibre optic cable comprises a fibre beam splitter, enabling the single laser 222 to provide light for a plurality of similar cytometer heads 226A, 226B. The fibre launch may be pig tailed and have no user adjustable parts and is stable to small environmental changes. The light is separated between the heads by a fibre beam splitter, which is a passive device with no moving parts and stable to small environmental fluctuations and has no user settings. The fibre emission may occur within the instrument such that no optical leverage exists and the entire instrument is more stable. Fibre coupling negates the requirement for optical tables and clamps to stabilise the coupling between laser and instrument. Fibre coupling negates the dust problem. The reduction in cooling fans, dust fans, ozone fans reduces vibration within the area and allows more stable cytometer operation. As no free space beams exist or may be easily accessed by personnel the laser safety issues are reduced and access to the room may be made simpler. Increased laser safety may reduce insurance premiums. Three photon lasers and harmonic crystals at wavelengths above 350nm create no significant ozone ands thus increase operator safety and negate the need for further filters, detection systems and controls, further reducing costs.

One method of sexing a sperm is given as an example. A sample of sperm is collected; sampling mechanisms are well known in the art and include dummy animals as is frequently used for cattle and manual manipulation of the prostate as is carried out for collection from elephants. The sperm may then be incubated with a dye. The sperm may be diluted. The material used to dilute the sperm may be selected such as to maintain the sperms environment. The dilutant may include egg.

The dye may be a type that will diffuse into the sperm and intercollate with the DNA. As female sperm comprise more DNA such sperm will be brighter. A second material may then be added. The second material may be a material that quenches the fluorescent emission of the first dye. This dye removes background signal. This dye may also remove the signal from dead sperm that have leaky or partial membranes as the second dye will the diffuse into the sperm. The sperm are then passed into a cell sorter. The sorter sends a jet of sperm through a nozzle. The nozzle may use a sheaf fluid, which allows hydrodynamic focusing and thus a narrow and well spatially defined jet of sperm. The nozzle maybe designed to make the jet into droplets such that each droplet could contain one sperm. The concentration of the sample should be such that on average no more than one in ten droplets cognition a sperm or some may cognition two or the nozzle may block. Preferably less than one in a hundred droplets contain sperm for this reason although this does reduce through put. Throughput is typically 10,000 sorted sperm a second although this may vary dramatically between species and specific animals.

The nozzle is also designed to rotate the droplet as it leaves the nozzle. The nozzle is also designed to charge the droplet with electrical energy.

The jet has a laser beam focused upon it at the position the droplets will have rotated ninety degrees. Thus the head of the sperm is facing a forward detector and the side of the sperm is facing a side detector. This allows analysis of the small intensity difference between male and female sperm, which is species specific but generally of the order of 2% to be measured as the sperm orientation can effect the direction of the emitted signal. This constraint also defines that the laser must be highly stabilised in amplitude.

The emitted signal is detected and analysed and a decision made of the sperm sex. The decision is a simple: does the signal suggest the sperm is of the required sex. If the result is positive the sample is collected, if negative the sample is not collected. Thus where laser stability or environmental variations due to cooling or environment control due to the lasers increase the uncertainty of the result there are less clear positives and significant sample is wasted. Where samples are from stud animals the sperm can have a very high market value and all sperm from the animal is utilised and more cannot be produced from the same animal only from an animal of lesser beneficial genetic characteristics, such that this wastage is a significant problem. Sorting occurs by switching a high voltage across charge plates in proximity to the jet of fluid. Typically a voltage of the order of 2000V is

switched which diverts the droplets path and allows it to be caught in a different sample pot.

The sperm is then kept chilled and packed into straws and frozen and the frozen straws distributed for veterinary surgeons to inseminate female animals.

The use of solid state lasers has significant advantages as they are simpler to stabilise in their intensity as they are smaller and more efficient. The reduced need for external cooling and air conditioning reduces vibration and thus improves the stability of the equipment. The use of fibre optics reduces the instability due to vibration and air currents when coupling free space. The reduced optical leverage when compared to free space beams makes the system more resistant to vibration and environmental changes.

The use of a beam splitter allows multiple heads to operate from a single laser. The use of placing the final non-linear element in the instrument head allows one laser to be used but allows operation by the user as if there was a laser to each head. The use of beam control such as an iris after the laser allows the laser to operate in gain saturated mode and be highly stable. The use of beam control such as an iris before any harmonic generator (when used) but after the laser allows the harmonic crystal to have an increased lifespan without increasing instability. The placement of the harmonic (when used) crystal in the instrument head allows all fibres to be infrared fibres, which are economic and low loss, unlike UV fibres. The reduction in mirrors reduces the losses. The use of holey fibres with high numerical aperture

reduces losses. When an Argon ion laser tube fails, which is typically of the order of, the entire laser cavity must be realigned when a new laser tube is fitted. This, is a time consuming and costly exercise requiring considerable skill. The realignment of the cavity require complete realignment of the laser to the cytometer which is a second skilled operation that may require a second set of personnel

When a solid state laser pump diode fails the operation is simple to replace and has no effect on the laser cavity alignment. The laser crystals are typically doped sappier with an exceptional lifespan but if a laser was terminally damaged by (for instance) lightening. The fibre connector may be disconnected and plugged into a new laser and that operation should take of the order of 15 seconds. Fibre optic connectors require almost no sill to disconnect and reconnect (although they may be locked for safety. The use of fibre optics means that the original laser does not have to be removed for the new one to be re-sited in its position. The new one may be sited in any position and the old one removed after the new one is installed.

The method described allows a spare laser to be kept ready and plugged in to any system that fails. The spare laser may be a on wheeled trolley to allow for it to be set up in any position in a workplace.

It is to be appreciated that the embodiments of the invention described above with reference to the accompanying drawings have been given by way of example

only and that modifications and additional components may be provided to enhance the performance of the apparatus.

The present invention extends to the above mentioned features taken singularly or in any combination.

Attention is directed to all papers and documents which are filed concurrently with or previous to this specification in connection with this application and which are open to public inspection with this specification, and the contents of all such papers and documents are incorporated herein by reference.

All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

Each feature disclosed in this specification (including any accompanying claims, abstract and drawings) may be replaced by alternative features serving the same, equivalent or similar purpose, unless expressly stated otherwise. Thus, unless expressly stated otherwise, each feature disclosed is one example only of a generic series of equivalent or similar features.

The invention is not restricted to the details of the foregoing embodiment(s). The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any

novel one, or any novel combination, of the steps of any method or process so disclosed.

Claims

1. Apparatus for characterizing an optical emission of a sample interrogated by a first optical pulse, which apparatus comprises a laser and an instrument, and in which the apparatus is configured such that the first optical pulse illuminates the sample, and the instrument is configured to characterize the optical emission.

2. Apparatus according to claim 1 and comprising a detector for detection of the optical emission, and in which the apparatus is configured such that the illumination and detection are confocal.

1. Apparatus according to claim 1 or claim 2 in which the instrument comprises a flow cytometer.

2. Apparatus according to claim 1 or claim 2 in which the instrument comprises a microscope.

3. Apparatus according to any one of the preceding claims in which the laser comprises a semiconductor laser.

4. Apparatus according to any one of claims 1 to 4 in which the laser is a Nd:Yag laser.

5. Apparatus according to any one of claims 1 to 4 in which the laser is a Nd glass laser.

6. Apparatus according to any one of claims 1 to 4 in which the laser is a fibre laser.

7. Apparatus according to claim 8 in which the fibre laser comprises a rare earth dopant selected from the group Ytterbium, Erbium, Neodymium, Praseodymium, Thulium, Samarium, Holmium and Dysprosium.

8. Apparatus according to any one of the preceding claims in which the laser is a mode-locked laser.

9. Apparatus according to any one of the preceding claims in which the laser comprises a continuous wave laser and an optical switch.

10. Apparatus according to any one of the preceding claims in which the laser emits the first optical pulse having a first wavelength.

11. Apparatus according to any preceding claim in which the laser emits a second optical pulse having a second wavelength, and in which the apparatus comprises at least one non-linear device for frequency conversion of the second optical pulse into the first optical pulse.

12. Apparatus according to claim 13 in which the non-linear device comprises a non-linear crystal.

13. Apparatus according to claim 14 in which the non-linear crystal is KTP.

14. Apparatus according to claim 13 in which the non-linear device comprises periodically poled lithium niobate.

15. Apparatus according to claim 13 in which the non-linear device comprises a parametric oscillator.

16. Apparatus according to claim 13 in which the non-linear device is selected from the group comprising an optical switch, a poled-fibre, a KTP or other crystalline wave guide, a non-linear optical loop mirror, a Kerr gate, an optical fibre, a non-linear amplifying loop mirror, and a non-linear optical modulator.

17. Apparatus according to claim 13 in which the non-linear device is a holey fibre.

18. Apparatus according to claim 19 in which the holey fibre comprises glass.

19. Apparatus according to claim 20 in which the glass is selected from the group comprising silica, a silicate glass, and a compound glass.

20. Apparatus according to any one of claims 19 to 21 in which the holey fibre comprises a core and a cladding, in which the cladding comprises a plurality of holes arranged around the core, and in which the core has a diameter less than 10um.

21. Apparatus according to claim 22 in which the core has a diameter less than 5um.

22. Apparatus according to claim 23 in which the core has a diameter less than 2um.

23. Apparatus according to any one of claims 19 to 25 in which the holey fibre comprises a dopant.

24. Apparatus according to claim 25 in which the dopant is selected from the group comprising Ytterbium, Erbium, Neodymium, Praseodymium, Thulium, Samarium, Holmium, Dysprosium, Tin, Germanium, Phosphorous, Aluminium, Boron, Antimony, Bismuth, Lead, a transition metal, and a semiconductor.

25. Apparatus according to claim 13 in which the non-linear device may be, or may comprise, a semiconductor optical amplifier.

26. Apparatus according to claim 13 in which the non-linear device comprises a lithium niobate wave guide.

27. Apparatus according to claim 28 in which the wave guide is a channel wave guide, or a planar wave guide.

28. Apparatus according to claim 13 in which the non-linear device comprises a periodically poled lithium niobate channel wave guide.

29. Apparatus according to claim 13 in which the non-linear device comprises a periodically poled lithium niobate planar wave guide.

30. Apparatus according to claim 13 in which the non-linear optical device is based on second-order (χ^2), or third-order (χ^3) non-linear effects including Self-Phase Modulation (SPM), Cross-Phase Modulation (CPM), Four-Wave Mixing (FWM), parametric frequency conversion, second harmonic generation, third harmonic generation, sum frequency generation, difference frequency generation, super continuum generation, cascaded second order effects, or some combination thereof.

31. Apparatus according to claim 13 in which the non-linear optical device is based on Raman and Brillouin effects, cross gain modulation or two photon absorption.

32. Apparatus according to any one of the preceding claims in which the apparatus comprises a pulse stretcher.

33. Apparatus according to claim 34 in which the pulse stretcher comprises an optical circulator and a fibre Bragg grating.

34. Apparatus according to claim 34 or claim 35 in which the pulse stretcher comprises a dispersive element.

35. Apparatus according to claim 34 in which the pulse stretcher comprises an optical fibre.

36. Apparatus according to any one of the preceding claims in which the apparatus comprises a pulse compressor.

37. Apparatus according to claim 38 in which the pulse compressor comprises a bulk grating.

38. Apparatus according to claim 38 or claim 39 in which the pulse compressor comprises an optical circulator and a fibre Bragg grating.

39. Apparatus according to claim 38 in which the pulse compressor comprises a dispersive element.

40. Apparatus according to any one of the preceding claims in which the apparatus comprises an optical amplifier.

41. Apparatus according to claim 42 in which the optical amplifier is an optical fibre amplifier comprises a rare-earth dopant selected from the group Ytterbium, Erbium, Neodymium, Praseodymium, Thulium, Samarium, Holmium, Dysprosium.

42. Apparatus according to claim 42 or claim 43 in which the optical fibre amplifier is one based on Erbium Ytterbium codoping.

43. Apparatus according to any one of the preceding claims in which the apparatus comprises a transmission fibre.

44. Apparatus according to claim 45 in which the transmission fibre is an optical telecommunication fibre.

45. Apparatus according to claim 45 or claim 46 in which the transmission fibre is configured to minimize dispersion.

46. Apparatus according to any one of claims 45 to 47 in which the transmission fibre is configured to minimize non-linear effects such as self phase modulation.

47. Apparatus according to any one of the preceding claims in which the apparatus comprises a first multiplexer.

48. Apparatus according to any one of the preceding claims in which the apparatus comprises a second multiplexer.

49. Apparatus according to any one of the preceding claims in which the laser is configured to emit short pulses.

50. Apparatus according to any one of the preceding claims in which the laser is configured to emit solitons.

51. Apparatus according to any one of the preceding claims in which the laser emits in the 1550nm telecommunication window.

52. Apparatus according to any one of the preceding claims in which the laser is configured to be remote from the sample.

53. Apparatus according to claim 54 in which the instrument comprises an optical detector configured to be remote from the sample.

54. An apparatus according to any preceding claim in which the apparatus comprises a sample handler for receiving the first optical pulse, in which the sample handler is connected to the laser by an optical cable having a length of at least 50 metres.

55. Apparatus according to claim 56, in which the optical cable has a length of at least 100metres.

56. Apparatus according to claim 56, in which the optical cable has a length of at least 1000metres.

57. Apparatus according to claim 49, in which the first multiplexer multiplexes the received optical pulse to a plurality of sample handlers.

58. Apparatus according to claim 1, in which the instrument comprises a non-linear crystal, which instrument is distant from the laser.

59. Apparatus according to any preceding claim in which the laser has a repetition rate set to ensure that each sample is illuminated by at least 50 laser flashes during its dwell time in the beam.

60. Apparatus according to claim 61 in which the laser has a repetition rate set to ensure that each sample is illuminated by at least 100 laser flashes during its dwell time in the beam.

61. Apparatus according to any preceding claim in which the laser is operated at or near saturation, the apparatus comprises a fibre for conveying light to a non-linear crystal and the magnitude of the light emitted to a sample is controlled by obstructing some of the light from the fibre from being incident on the non-linear crystal.

62. An apparatus according to claim 1, in which there is provided means for exciting the sample by multiphoton excitation.

63. An apparatus according to claim 64 in which the multiphoton excitation is second order multiphoton excitation.

64. An apparatus according to claim 64 in which the multiphoton excitation is third order multiphoton excitation.

65. An apparatus according to any one of claims 64 to 66 in which the multiphoton excitation is configured to occur within an effective area that is smaller than the area that the laser is focussed onto within the sample.

66. An apparatus according to any one of claims 64 to 67 in which the sample is defined by an effective wavelength characterised by single photon excitation and in which the sample is not penetrative to illumination at the effective wavelength, and in which the method comprises illuminating the sample at a wavelength that is penetrative to the sample using multiphoton excitation.

67. An apparatus according to claim 68 in which the effective wavelength is less than 350nm, wherein the laser delivers UV light without formation of ozone.

68. An apparatus according to any one of claims 64 to 69 in which the instrument comprises a detector, and in which the detector has a higher gain at wavelengths emitted by the sample under test than the wavelength of the multiphoton excitation.

69. An apparatus according to any one of claims 64 to claim 70 in which the wavelength of the multiphoton excitation is in the region of 1500-1600nm.

70. An apparatus according to any one of claims 64 to claim 70 in which the wavelength of the multiphoton excitation is in the region of 970nm to 1140nm.

71. An apparatus according to any one of claims 64 to claim 70 in which the wavelength of the multiphoton excitation is in the region 750-900nm.

72. An apparatus according to any one of claims 64 to 73, in which the apparatus comprises a probe pulse to generate emission from a sample.

73. An apparatus according to any one of claims 64 to 74 in which the apparatus further comprises a short pulse generator for generating a short pulse is used to generate multiphoton excitation of the sample.

74. An apparatus according to claim 75 and including a pulse stretcher for stretching the short pulse.

75. An apparatus according to claim 76 and including an amplifier for amplifying the stretched short pulse, and a compressor for compressing the amplified stretched short pulse.

76. An apparatus according to any one of claims 75 to 77 in which the short pulse is a soliton.

77. An apparatus according to any one of claims 74 to 78 in which the sample is remote from the laser.

78. A sperm sexing apparatus comprising apparatus for characterizing an optical emission of a sample according to any preceding claim.

79. A method for analysing a sample comprising providing a laser, a sample, and an instrument, and exciting the sample by multiphoton excitation.

80. A method according to claim 81 in which the multiphoton excitation is fourth order multiphoton excitation.

81. A method according to claim 81 in which the multiphoton excitation is fifth order multiphoton excitation.

82. A method according to any one of claims 81 to 83 in which the multiphoton excitation is configured to occur within an effective area that is smaller than the area that the laser is focussed onto within the sample.

83. A method according to any one of claims 81 to 84 in which the sample is defined by an effective wavelength characterised by single photon excitation and in which the sample is not penetrative to illumination at the effective wavelength, and in which the method comprises illuminating the sample at a wavelength that is penetrative to the sample using multiphoton excitation.

84. A method according to claim 85 in which the effective wavelength is less than 350nm, wherein the laser delivers UV light without formation of ozone.

85. A method according to any one of claims 81 to 86 in which the instrument comprises a detector, and in which the detector has a higher gain at wavelengths

emitted by the sample under test than the wavelength of the multiphoton excitation.

86. A method according to any one of claims 81 to claim 87 in which the wavelength of the multiphoton excitation is in the region of 1500-1600nm.

87. A method according to any one of claims 81 to claim 87 in which the wavelength of the multiphoton excitation is in the region of 970nm to 1140nm.

88. A method according to any one of claims 81 to claim 87 in which the wavelength of the multiphoton excitation is in the region 750-900nm.

89. A method for analysing a sample according to any one of claims 56 to 68 and including the step of providing a probe pulse to generate emission.

90. A method for analysing a sample according to any one of claims 56 to 68 in which a short pulse is used to generate multiphoton excitation of the sample.

91. A method according to claim 70 and including the step of stretching the short pulse.

92. A method according to claim 70 and including the steps of stretching the short pulse, amplifying the stretched short pulse, and compressing the amplified stretched short pulse.

93. A method according to any one of claims 70 to 72 in which the short pulse is a soliton.

94. A method according to any one of claims 70 to 73 in which the sample is remote from the laser.

95. A method for analysing samples comprising the step of generating solitons, stretching the solitons, and amplifying the stretched solitons.

96. A method for analysing samples according to any one of claims 56 to 75 in which the sample is selected from the group comprising a biological material, a tissue sample, a protein, a cell, a section of a protein, a section of DNA, a section of RNA, a chromosome, a gene, a sperm cell, an antibody, an animal and a person.

97. A method for sexing sperm of an animal comprising using an apparatus according to any one of claims 1 to 80.

98. A method according to claim 99 in which the animal is livestock comprising one of cattle, pigs, and horses.

99. A method for artificial insemination according to claim 99 or claim 100 comprising inseminating a female animal with the sperm from the same species.

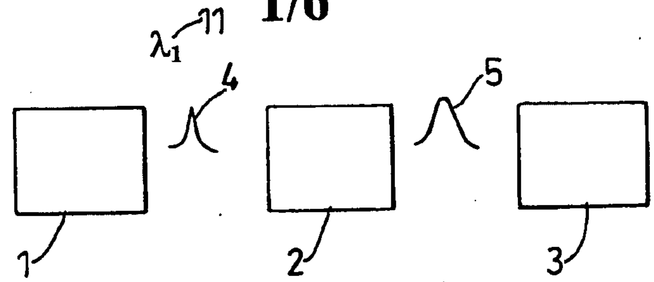

Fig. 1

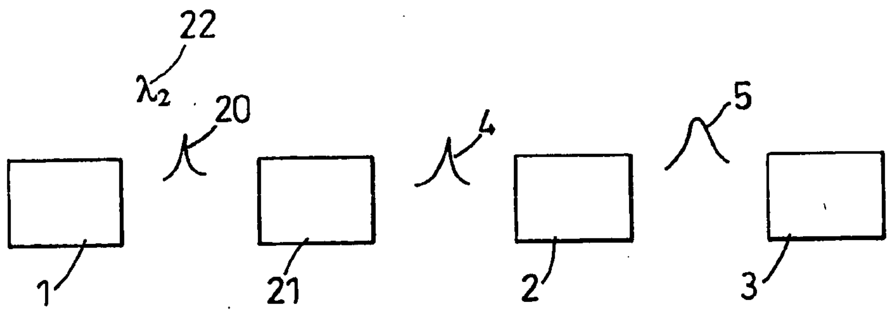

Fig. 2

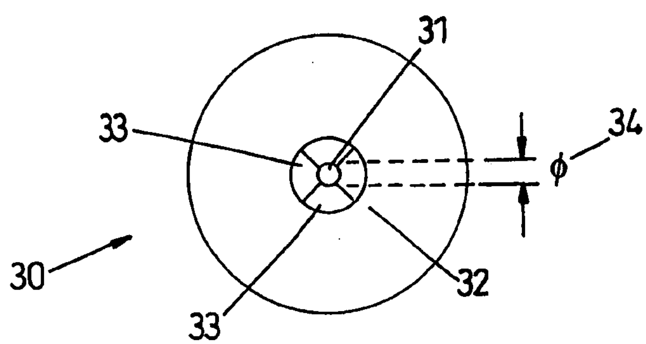

Fig. 3

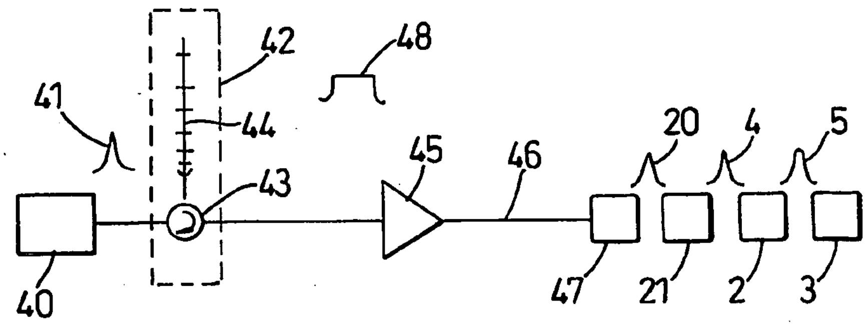
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Fig. 4

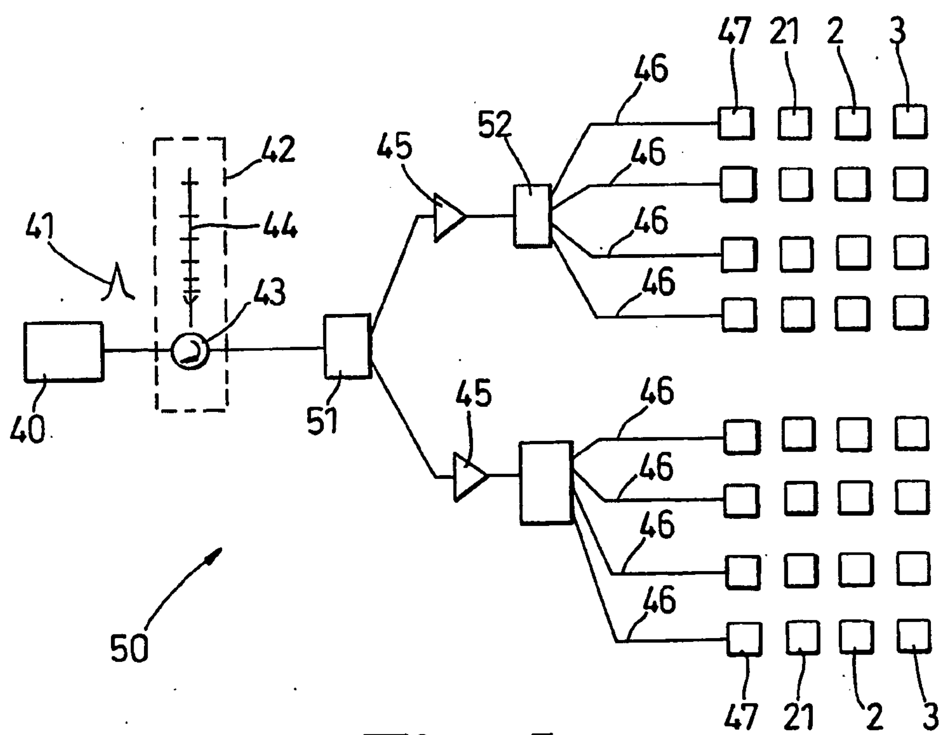
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Fig. 5

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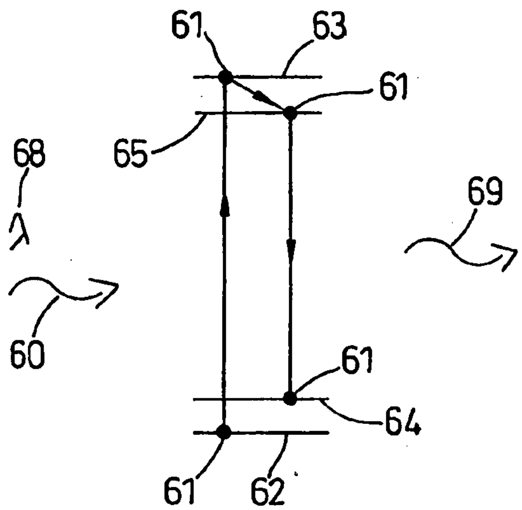

Fig. 6

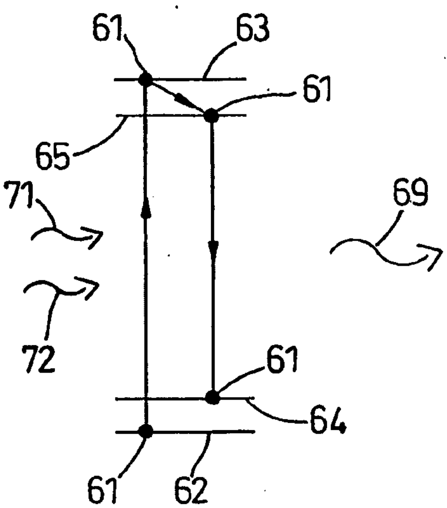

Fig. 7

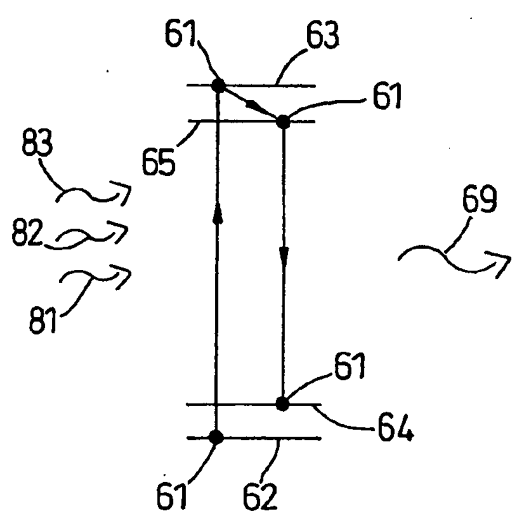

Fig. 8

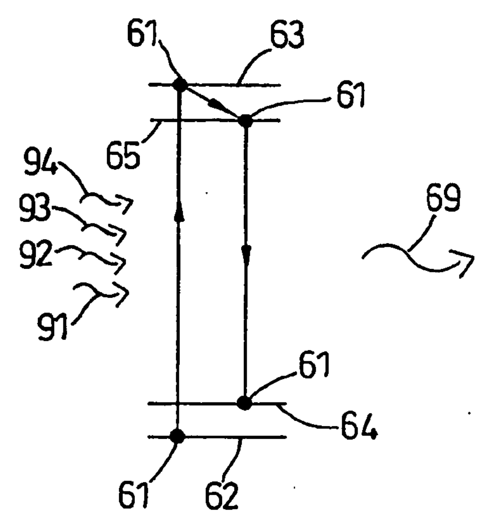

Fig. 9

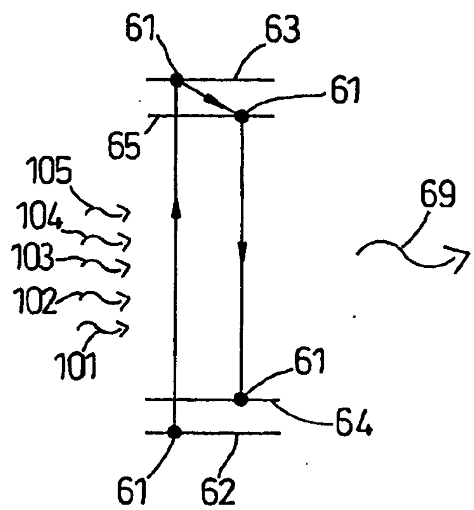

Fig. 10

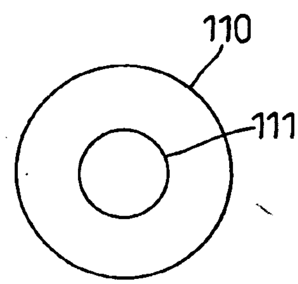

Fig. 11

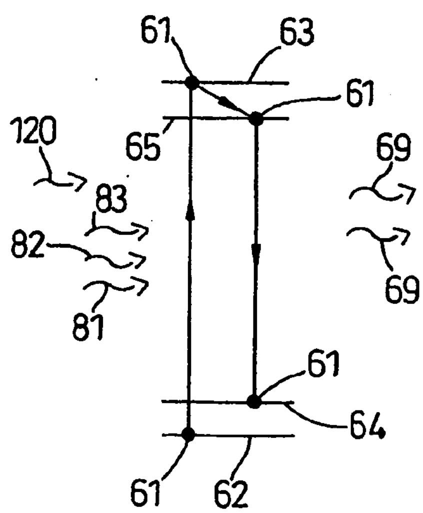

Fig. 12

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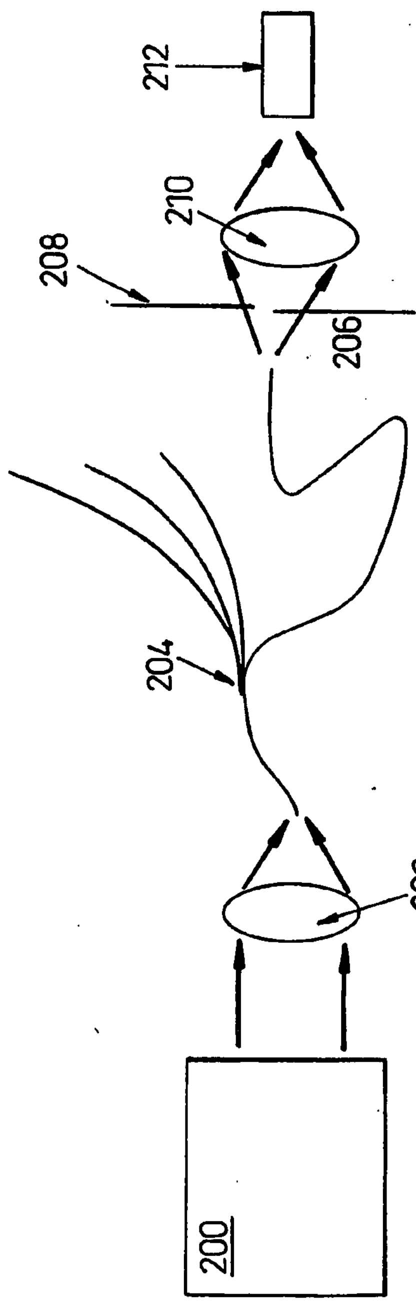

Fig. 13

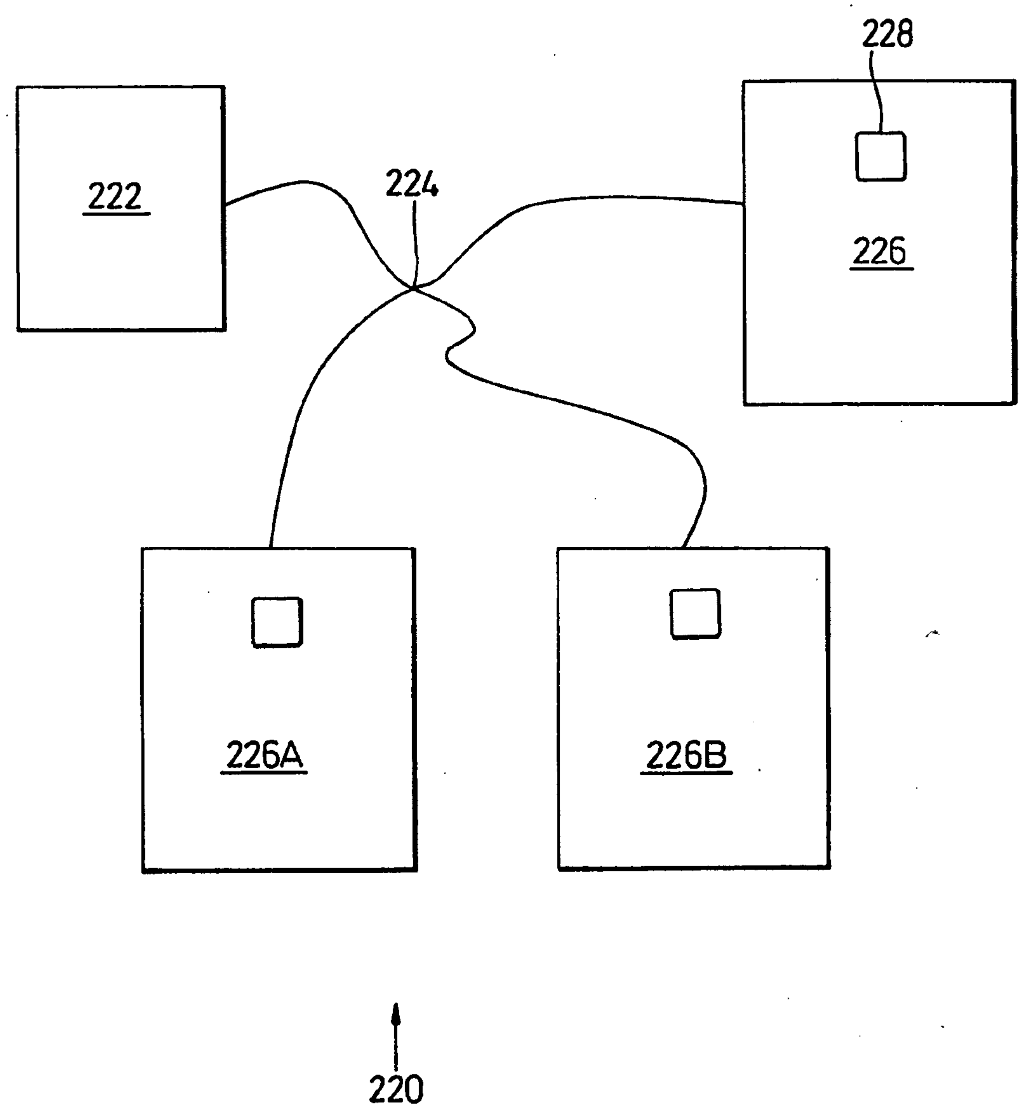

Fig. 14